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Review

What Is the Difference Between *Helicobacter pylori*-Associated Dyspepsia and Functional Dyspepsia?

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Advances in basic and clinical research have revealed that *Helicobacter pylori* (*H. pylori*) infection plays an important role in the development of gastroduodenal dysmotility and hypersensitivity, as also in dyspepsia symptoms. In addition, recent studies have proposed an inflammation-immunological model for the pathogenesis of functional dyspepsia. Since *H. pylori* is the major microbe that provokes a gastroduodenal inflammatory response, it should not be overlooked when considering the pathophysiology of dyspepsia symptoms. In fact, population-based studies have demonstrated that *H. pylori* is detected more frequently in dyspepsia patients. However, although many clinical studies tried to reveal the association of *H. pylori* infection with gastric motility dysfunction or hypersensitivity, the results have been conflicting. On the other hand, many etiological features were revealed for the development of *H. pylori*-associated dyspepsia, such as abnormal ghrelin or leptic secretion, altered expression of muscle-specific microRNAs, and duodenal inflammatory cell infiltration. In addition, therapeutic strategy for *H. pylori*-associated dyspepsia would be different from *H. pylori*-negative functional dyspepsia. This review focuses the issue of whether *H. pylori*-associated dyspepsia should be considered as a different disease entity from functional dyspepsia.
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Key Words
Duodenum; Ghrelin; *Helicobacter pylori*; MicroRNAs

Introduction

Functional dyspepsia (FD) is a syndrome characterized by chronic and recurrent gastroduodenal symptoms in the absence of any organic or metabolic disease that is likely to explain the symptoms.[1,2] FD is considered to be important to public health, because it is remarkably common, can be disabling, and can pose a major social and economic burden.[3] Since FD is a highly heterogeneous disorder, numerous pathophysiological mechanisms, such as gastroduodenal motor dysfunction, visceral hypersensitivity, central nervous system dysfunction, *Helicobacter pylori* (*H. pylori*) infection and psychosocial factors have been suggested to play a role in the development of FD.

Although numerous epidemiological trials have suggested a higher prevalence of *H. pylori* infection in FD patients, the results have been conflicting.[4] Results of a meta-analysis showed that the prevalence of *H. pylori* infection was greater in patients with dys-

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pepsia than in controls, with an odds ratio of 2.3 (95% CI, 1.9-2.7).[5] Although this result seems to support the role of *H. pylori* infection in the pathogenesis of dyspepsia, it appears that some of the studies that were included in the analysis were biased by the selection of controls not properly matched for age, socioeconomic status and ethnic background.[4]

However, recent studies have revealed a subset of patients who developed FD after an episode of gastrointestinal infection. These studies, proposing the concept of post-infectious FD, suggest that an inflammation-immunological circuit also plays an important role in the development of FD.[6] It is generally well-recognized that the major cause of gastroduodenal inflammation is *H. pylori* infection.[7] Since *H. pylori* induces activation of a complex and fascinating cytokine and chemokine network in the gastric mucosa,[8] it is of little surprise that *H. pylori* infection has been implicated in the pathogenesis of dyspepsia.

For this reason, one of the major research interest is the difference between *H. pylori*-associated dyspepsia and other functional dyspepsia.[9] In this review article, FD in patients with a present or even past history of *H. pylori* infection is defined as a different disease entity (*H. pylori*-associated dyspepsia [HpD]) from FD, especially by focusing on the etiological insight of HpD, and then discusses the therapeutic strategy of HpD.

Influence of *Helicobacter pylori* Infection on Dyspepsia Symptoms and the Gastric Functions

A lot of clinical evidences have been published to investigate whether *H. pylori* infection is involved in gastric motility disorders and visceral hypersensitivity. However, all of these studies were small-scale studies, and the results were conflicting. Few studies have shown the association between gastric visceral hypersensitivity and *H. pylori* infection. Thumshirn et al[10] compared gastric motor and sensory functions in 17 patients with FD and 16 asymptomatic controls, and reported that *H. pylori* infection did not appear to influence gastric accommodation, but was associated with hypersensitivity in FD patients. On the other hand, some researchers were able to show the association between gastric motility dysfunction and *H. pylori* infection. Mearin et al[11] investigated the symptomatic pattern in 27 *H. pylori*-positive and 23 *H. pylori*- negative patients with FD, and showed that FD patients with *H. pylori* infection presented no distinctive symptoms in comparison with their *H. pylori*-negative counterparts, and that *H. pylori* infection was associated with diminished postprandial antral motility, but did not increase the perception of gastric distension. Tucci et al[12] evaluated the *H. pylori* infection status, histological features of the gastric mucosa, and the gastric motor and secretory functions in 45 consecutive patients with FD. *H. pylori* infection was found in 60% of FD patients, as compared with 33% of the 15 healthy controls. No difference was detected in the basal or stimulated gastric acid secretion between the FD patients and healthy controls. Gastric emptying was significantly delayed in FD patients as compared with that in healthy controls after adjustments for age and sex. Delayed gastric emptying was associated with a low frequency of *H. pylori* infection, female gender and young age. Epigastric pain or burning and postprandial fullness were more severe in patients with *H. pylori* infection and in those with delayed gastric emptying, respectively. Saslow et al[13] compared 8 *H. pylori*- positive and 8 *H. pylori*-negative asymptomatic subjects, and showed that *H. pylori* infection reduced accommodation, but had no effect on the overall sensation or motor functions of the stomach. However, some studies showed that *H. pylori* infection did not affect gastric motility or hypersensitivity. Leontiadis et al[14] evaluated 23 FD patients and 17 controls, and showed that although gastric emptying was delayed in FD patients, the gastric emptying rate was not associated with the *H. pylori* infection status, and was also not affected by eradication of the infection. Chang et al[15] compared 22 *H. pylori*- negative patients and 38 *H. pylori*- positive patients with FD, and showed that the *H. pylori* infection status appeared to have no influence on the incidence of delayed gastric emptying of digestible and indigestible solids.

Although the results of several clinical studies suggest that *H. pylori* infection may play a role in the development of FD, the precise pathogenesis of HpD could not be elucidated. Since gastric dysmotility and visceral hypersensitivity are induced by a number of confounding factors, such as diet, smoking and psychosocial stress, the association of *H. pylori* infection with gastric sensation or motor dysfunction might be difficult to be revealed only by clinical studies. A large-scale clinical study controlled for all of these factors would be difficult to design. Thus, novel biological markers for HpD other than gastric dysmotility and hypersensitivity must be identified. On next section, therefore, the possible pathophysiology of HpD will be reviewed.

Pathophysiological Link Between *Helicobacter pylori* Infection and Dyspepsia

Traditionally, gastric acid hypersecretion induced by *H. pylori*

infection of the gastric antral mucosa has been considered to play a role in the development of dyspepsia. About 10%-15% of patients with *H. pylori* infection show antral-predominant gastritis, which results in gastric acid hypersecretion.[16] In these patients, *H. pylori* induced a decrease in somatostatin secretion in the antral gland area, leading to an increase in the release of gastrin and subsequently to a rise in acid secretion.[17] This mechanism is also considered to underlie the development of duodenal ulcer. These phenomena are reversible, since normal feedback control of gastrin secretion is restored after *H. pylori* eradication.[17,18]

However, a few studies investigating the association between the severity of histological gastritis and that of dyspepsia symptoms yielded different results. Turkkan et al[19] reported that dyspepsia symptom scores were higher in patients with mild or moderate chronic inflammation of the corpus and antrum than in those with severe chronic inflammation, although the difference did not reach statistical significance. In studies conducted by Joshi et al[20] and Pereira-Lima et al,[21] no relationship was found between the severity of histological gastritis and the severity of the dyspeptic symptoms. Czinn et al[22] found a relationship between epigastric pain and the severity of inflammation. Similarly, van der Schaar et al[23] also found an indirect relationship between the severity of symptoms and the severity of inflammation of the corpus. From these results, we could not reach any definitive conclusion about the association of severity of gastritis or amount of gastric acid secretion with severity of the dyspepsia symptoms.

Ghrelin, which is produced and secreted by the A-like cells of the oxyntic glands of the stomach, has a well-established role in increasing appetite and food intake and in stimulating gastric emptying and acid secretion.[24-28] These functions are mediated, at least in part, via vagal nerve pathways.[29,30] In gastroduodenal mucosal injury, the levels of plasma ghrelin increased in response to the physiological demand for the purpose of gastroduodenal cytoprotection.[31,32] However, in the presence of *H. pylori*-induced severe gastric mucosal atrophy, the plasma ghrelin concentrations shifted to lower levels.[33-36] Taken together, *H. pylori* infection may induce gastric motor dysfunction and reduce appetite with suppressed ghrelin secretion. Therefore, this peptide may play a role in the onset of FD, especially HpD. In fact, alterations of the plasma ghrelin levels have been reported in FD patients, which frequently correlated with the FD symptom score.[37-39] Some studies showed that plasma ghrelin levels were significantly lower in patients with dysmotility-like FD.[28,37] Concerning the active ghrelin levels, they were also decreased in patients with postprandial fullness and/or early satiation,[40] whereas similar between dysmotility-like FD patients and healthy controls.[37] Moreover, recent study showed that repeated ghrelin administrations had stimulatory effects on food intake in FD patients.[41] However, the opposite results, such as enhanced ghrelin levels in FD patients, were also reported.[38,42] Leptin is also produced in the stomach, and activates vagal nerve ternimals, reduces appetite and increases mucin secretion.[43] Leptin may also play a role in the onset of FD, since patients with dysmotility-like dyspepsia have been reported to show higher serum concentrations of leptin.[44] On the other hand, serum leptin levels and expression of leptin mRNA in the gastric mucosa was enhanced in *H. pylori*-positive patients,[44,45] suggesting that *H. pylori* infection may reduce appatite with enhanced leptin secretion. The circulatory levels of ghrelin and leptin in HpD patients have not yet been investigated, warranting future research.

We recently investigated the role of microRNAs (miRNAs) in gastric motility disorders associated with *H. pylori* infection,[46] and the results provided a novel insight into the molecular pathogenesis of HpD. Histologic examination showed prominent thickening of the muscular layer of the gastric corpus in *H. pylori*-infected mice. In addition, gastric emptying was significantly accelerated in *H. pylori*-infected mice. The miRNA expression profile revealed that the muscle-specific miRNAs, *miR-1, miR-133a* and *miR-133b*, were downregulated in the stomach of *H. pylori*- infected mice. The expression levels of histone deacetylase 4 and serum response factor, which are target genes of *miR-1* and *miR-133* known to enhance muscular hyperproliferation, were increased. Taken together, chronic *H. pylori* infection downregulates the expressions of muscle-specific miRNAs and upregulates the expression of histone deacetylase 4 and serum response factor, which might cause hyperplasia of the muscular layer of the stomach and deregulation of gastric emptying in mice. Further human studies will be necessary to validate the association between aberrant expression of muscle-specific miRNAs in the muscular layer of the stomach and HpD.

Duodenum - A Crossroad Between *Helicobacter pylori* and Dyspepsia

Recent studies have emerged implicating abnormal motor and autonomic responses in the duodenum perhaps triggering functional responses, including pain and abnormal gastric emptying. Increased duodenal acid exposure has been reported in patients with dyspepsia symptoms. At the level of the duodenum, abnormalities may exist in the stimulus intensity, mucosal

mRNA expression, biosynthesis, release or inactivation of the mucosal mediators, or in the receptor expression on the afferent nerve endings.[47]

Furthermore, Talley et al[48] proposed that changes in the duodenal eosinophil count might be an underlying feature of FD. They also showed that eosinophils were significantly increased in both the bulb and second portion of the duodenum in FD, whereas increase of the mast cells in the second portion of the duodenum was noted in irritable bowel syndrome (IBS).[49,50] A link between eosinophils (and other inflammatory cells) and FD would have therapeutic implications. Eosinophils are critically dependent on the cytokine IL-5 for their maturation in the bone marrow, which also influences eosinophil migration and survival. Kindt et al[51] reported that stimulated lymphocyte expression of IL-5 and IL-13 was enhanced, whereas stimulated monocytic IL-12 and lymphocytic IL-10 expression were reduced in both FD and IBS. Based on these findings, anti-inflammatory agents, possibly including novel biologics such as anti-IL-5 humanized antibodies, could be explored as a possible therapeutic candidates for FD.

Active duodenitis has been reported to be more common in patients with *H. pylori* infection.[52] Genta et al[52] reported that *H. pylori* was detected in the gastric metaplastic epithelium of 67.6% of patients with active inflammation of the duodenum. On the other hand, *H. pylori* infection is well-known to cause eosinophil infiltration of the gastric mucosa.[53] Taken together, *H. pylori* might be one of the causes of duodenal eosinophilia, as well as of the onset of dyspepsia symptoms.

In addition, Gargala et al[54] reported that the number of intraepithelial lymphocytes in the duodenal mucosa was significantly greater in *H. pylori*-positive FD patients than in healthy controls, but not different between *H. pylori*-negative FD patients and healthy controls. The expressions of CD95/Fas and HLA-DR-expressing $CD3^+$ lymphocytes were lower in *H. pylori*-negative FD patients than in healthy controls. These findings suggest that the phenotypic characteristics of intraepithelial lymphocytes may be different between HpD and *H. pylori*-negative FD.

Treatment for *Helicobacter pylori*-Associated Dyspepsia

Although a number of clinical trials have assessed the efficacy of *H. pylori* eradication for the treatment of FD, the studies drew different conclusions. However, it is quite clear that *H. pylori* eradication treatment is effective in at least a subset of patients with FD.[7,55-58] According to a meta-analysis of randomized controlled trials to determine the effect of *H. pylori* eradication on dyspepsia symptoms, *H. pylori* eradication therapy appears to have a small but statistically significant effect in HpD.[59] Harvey et al[60] showed that *H. pylori* eradication gave cumulative long-term benefit, with a continued reduction in the development of dyspepsia severe enough to require a consultation with a general practitioner up to at least 7 years.

The efficacy for patients with HpD in Asia would be different from those in Western countries, since Asian population differs from the Western population in many respects, such as prevalent *H. pylori* strains, including cagA gene polymorphisms, levels of acid secretion in the stomach and the severity or pattern of gastritis.[58,61] In fact, Gwee et al[62] showed that the patients with FD in Asia would have a benefit from treatment for *H. pylori* infection with as much as a 13-fold increased chance of symptom resolution following its eradication in a double blind, randomized and placebo-controlled trial in Singapore-based Asian population.

There is no evidence of treatment for HpD patients after the successful eradication of *H. pylori*. At present, acid suppression is a frequently used first-line therapy for FD. A meta-analysis of randomized controlled trials of proton pump inhibitors (PPIs) for FD reported that this class of agents was superior to placebo.[63] However, much of this benefit may be explained by the presence of concomitant unrecognized gastroesophageal reflux disease (GERD). Xiao et al[64] showed that the prevalence of pathologic esophageal acid reflux without typical reflux symptoms (silent reflux) was 31.7% in FD patients. In addition, PPIs were effective in 83.1% of FD patients with silent reflux, and in 54.3% of those without silent reflux. On the other hand, inverse associations are observed between the presence of *H. pylori* infection and GERD, because of the reduction in gastric acid production by *H. pylori* colonization of the gastric mucosa.[65,66] This suggests that the efficacy of PPIs in HpD may be weaker than that in *H. pylori*-negative FD, which may show strong overlap with GERD.

On the other hand, a gastro-protective agent for chronic gastritis would be a therapeutic candidate for HpD. Rebamipide, a gastro-protective anti-ulcer drug, has been used for the improvement of dyspepsia symptoms in Japan, Korea, China and some other countries. Rebamipide is known to suppress gastric mucosal inflammation, which is thought to be related to its activity in the inhibition of superoxide anion production from neutrophils and scavenging hydroxyl radicals.[67,68] Rebamipide administration after *H. pylori* eradication could promote the restoration of atro

phic mucosa in Mongolian gerbils.[69] Chitapanarux et al[70] reported that rebamipide treatment improved symptom, endoscopic and histologic features of chronic gastritis in patients with dyspepsia symptoms refractory to PPIs. Talley et al[71] reported a double-blind, placebo-controlled and multicenter study of rebamipide for the treatment of FD patients with or without *H. pylori* infection. Although a significant improvement of individual symptoms at 8 weeks was not detected, the ratio of patients who requested usage of the study medication again was greater in the rebamipide groups compared with the placebo group in *H. pylori*-positive patients. During the planning of this study, it was originally projected that a sample size of 100 patients per treatment group would be sufficient to detect a difference in response rate of approximately 20% between the rebamipide treatment group and the placebo treatment group with 80% power at the 0.05 significance level. However, because of the slow patient recruitment and unexpected budget constraints, the trial had stopped prior to completion of enrollment. Based on the enrolled population of approximately 50 patients per arm in the *H. pylori*-negative study and 30 patients per arm in the *H. pylori*-positive study, the detectable differences would be 30% and 40%, respectively. The 30% superiority over the placebo would be non-realistic hurdle for any medication for FD. Miwa et al[72] also reported a double-blind, placebo-controlled and single-center study of rebamipide for the treatment of FD patients. Although the mean changes in overall symptoms after 4 weeks of treatment were not significantly different between the rebamipide and placebo treatment groups, the improvement in symptom score was significantly greater in the rebamipide group for bloating, belching and pain or discomfort that was relieved after a meal. Social restriction and pain intensity were also improved in the rebamipide group. The ratio of subjects with *H. pylori* infection were 54.1% in the rebamipide group and 42.4% in the placebo group. However, they did not perform subanalysis by *H. pylori* status as the number of subjects was rather small. As rebamipide has an anti-inflammatory effect, it might be effective for HpD, but not for FD patients without gastritis. However, there is not enough evidence for the efficacy of rebamipide for dyspepsia symptoms of HpD patients.

Therefore, the efficacy of all the existing medical treatment, including a gastro-protective agent, for FD should be re-evaluated for HpD and *H. pylori*-negative FD. Well-designed studies to investigate a suitable therapeutic strategy for HpD are needed.

Conclusions

Several mechanisms have been postulated for the development of HpD. Some of these mechanisms would be reversible, while others might not. Therefore, it would be reasonable that the *H. pylori* "test-and-treat" strategy is not effective in all HpD patients, but is effective in only a subset of HpD patients. *H. pylori* infection evokes significant inflammatory changes, not only in the gastric mucosa, but also in the gastric muscular layer as well as in the duodenum. However, most patients with *H. pylori* infection do not have any symptoms. We therefore need to conduct further investigation about the true relationship between dyspepsia symptoms and *H. pylori* infection to determine whether there might be identifiable risk factors for the onset of symptoms.

When the Rome III criteria were developed, the role of *H. pylori* infection in FD was controversial. Now, however, the pathophysiology underlying disturbances of gastroduodenal motor or sensory function and dyspepsia symptoms caused by *H. pylori* infection is gradually being elucidated. Therefore, when HpD is considered as an organic disease and as a different disease entity from FD, these conflicting results of previous studies might become more comprehensible. Further studies will be necessary to determine whether HpD should be separated from FD. In addition, the differences in the therapeutic strategies between HpD and *H. pylori*-negative FD are also necessary to be investigated in the future.

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Enhancement of Amoxicillin Resistance after Unsuccessful *Helicobacter pylori* Eradication▽

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A high rate of resistance (49.5 to 72.7%) to amoxicillin (AMX) was observed in *Helicobacter pylori* after two or three unsuccessful eradication attempts. Unsuccessful eradication regimens significantly increase resistance to not only clarithromycin (CLR) and metronidazole (MNZ) but also AMX.

Currently available eradication regimens for *Helicobacter pylori* are triple-drug combination regimens comprising a proton pump inhibitor (PPI) and two antibiotic drugs, and clarithromycin (CLR), metronidazole (MNZ), and amoxicillin (AMX) are commonly used antibiotics (12). Although *H. pylori* bacteria easily become resistant to CLR and MNZ, *H. pylori* has been thought to seldom become resistant to AMX (6). In the present study, the resistance rates after unsuccessful eradication attempts were examined.

A total of 343 patients (189 males and 154 females; mean age, 55.8 years) with *H. pylori* infection were enrolled between September 2004 and October 2010. *H. pylori* infection was defined by *H. pylori* culture positivity. Of the total, 22 patients had no history of antibacterial therapy for eradication, 211 patients had one treatment failure, 99 patients had two treatment failures, and 11 patients had three treatment failures (first-line treatment, triple therapy with CLR [800 mg/day], AMX [1,500 mg/day], and PPI for 7 days; second-line treatment, triple therapy with MNZ [500 mg/day], AMX [1,500 mg/day], and PPI for 7 days; third-line treatment, triple therapy with fluoroquinolone [levofloxacin, 400 mg/day; gatifloxacin, 400 mg/day; or sitafloxacin, 400 mg/day], AMX [2,000 mg/day], and PPI for 7 days) (8, 13). All patients underwent esophagogastroduodenoscopy and gastric biopsy for bacterial culture 6 to 12 months after the eradication failure at Keio University Hospital and National Tokyo Medical Center.

TABLE 1. Eradication failures and resistance rates

Agent	Prior treatment	% resistance (no. of resistant strains/no. tested)[a]			MIC of agent		
		With AMX MIC (μg/ml) of:		Other resistance			
		≥0.06	≥0.5		50%	90%	Range
AMX	None	13.6 (3/22)	0 (0/22)		<0.015	0.06	<0.015–0.12
	One failure	26.5 (56/211)	0.9 (2/211)		<0.015	0.12	<0.015–0.5
	Two failures	49.5 (49/99) ++ ###	6.1 (6/99) #		0.03	0.25	<0.015–4
	Three failures	72.7 (8/11) +++ ##	18.2 (2/11) ##		0.12	0.5	<0.015–4
CLR	No treatment			9.1 (2/22)	0.03	0.25	<0.015–8
	One failure			89.6 (189/211) +++	16	32	<0.015–64
	Two failures			88.8 (88/99) +++	16	32	<0.015–64
	Three failures			72.7 (8/11) +++	16	64	<0.015–64
MNZ	None			13.6 (3/22)	2	8	0.25–32
	One failure			4.7 (10/211)	1	2	0.5–32
	Two failures			72.7 (72/99) +++ ###	16	64	1–64
	Three failures			72.7 (8/11) ++ ###	16	32	4–32

[a] AMX resistance, MIC ≥ 0.06 μg/ml; AMX high-level resistance, MIC ≥ 0.5 μg/ml; CLR resistance, MIC ≥ 1μg/ml; MNZ resistance, MIC ≥ 8 μg/ml. ++, $P < 0.01$ versus results for nontreatment group; +++, $P < 0.001$ versus results for nontreatment group; #, $P < 0.05$ versus results for one-failure group; ##, $P < 0.01$ versus results for one-failure group; ###, $P < 0.001$ versus results for one-failure group.

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TABLE 2. Substitutions in penicillin binding protein 1 of *H. pylori* strains

Strain or group (n^a)	AMX MIC (μg/ml)	Substitution at penicillin binding protein 1 position:						
		374	406	414	535	593	599	601
KS0461	4				Asp		Gly	Gly
KS0478	0.5				Asp			
KS0487	0.5	Leu		Arg				
KS0439	0.25		Ala			Ala		
KS0476	0.25				Asp		Gly	
KS0470	0.25				Asp			
KS0444	0.12		Ala			Ala		
KS0464, KS0479	0.12				Asp			
KS0493	0.12						Pro	
KS0434	0.06		Ala					
KS0466, KS0491	0.06				Asp			
KS0503	0.06			Arg				
KS0502	0.06							
AMX-susceptible strains (15)	≤0.03	Val	Glu	Ser	Asn/Asp[b]	Thr	Ala	Val

[a] *n*, no. of strains.
[b] $535_{Asn \rightarrow Asp}$ was detected in the amoxicillin-susceptible strains KS0447, KS0452, and KS0467.

Susceptibilities of *H. pylori* isolates to AMX, CLR, and MNZ were determined by the agar dilution method according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI) (1, 7). Isolates were considered resistant to MNZ if the MIC of the drug was ≥8 μg/ml and to CLR if the MIC was ≥1 μg/ml (9). For AMX, the interpretive standard (susceptible, ≤0.03 μg/ml) established by the Japanese Society of Chemotherapy was used (3). Isolates were defined as high-level resistant and resistant to AMX if the MIC was ≥0.5 μg/ml and ≥0.06 μg/ml, respectively (11), in this study. Differences between groups were compared by Fisher's exact test or the chi-squared test.

The rates of resistance to AMX in the groups with no history of eradication treatment, one treatment failure, two treatment failures, and three treatment failures were 13.6%, 26.5%, 49.5%, and 72.7%, respectively. The high-level rates of resistance to AMX in the group with no history of eradication treatment, one treatment failure, two treatment failures, and three treatment failures were 0%, 0.9%, 6.1%, and 18.2%, respectively (Table 1). The rates of resistance to AMX in the group with two treatment failures and that with three treatment failures were significantly higher than that in the group with no history of eradication treatment and that with one treatment failure. To the best of our knowledge, the present study is the first to report the increase in rates of resistance to AMX after unsuccessful *H. pylori* eradication.

The MIC_{90} of AMX showed 2-fold increases with every eradication failure. The MIC_{90} of CLR showed a 128-fold increase after triple therapy with CLR, AMX, and PPI, and the MIC_{90} of MNZ showed a 32-fold increase after triple therapy with MNZ, AMX, and PPI (Table 1). While the 23S rRNA point mutation is a main cause of CLR resistance (4) and the single mutation of *rdxA* or *frxA* is one of the main causes of MNZ resistance (5), multiple mutations in penicillin binding protein 1 (PBP1) would contribute to a greater increase in the level of AMX resistance (11) and then could result in a gradual increase in AMX resistance.

TABLE 3. Amoxicillin resistance rate and susceptibility to clarithromycin and metronidazole

Resistance	% amoxicillin resistance (no. of resistant strains/no. tested)[a]	MIC	
		50%	90%
CLR susceptible, MNZ susceptible	13.6 (6/45)	<0.015	0.06
CLR resistant, MNZ susceptible	32.2 (66/205) +	0.03	0.12
CLR susceptible, MNZ resistant	45.5 (5/11) +	0.03	0.5
CLR resistant, MNZ resistant	48.8 (40/82) # +++	0.03	0.25

[a] Amoxicillin resistance: MIC ≥ 0.06 μg/ml. +, $P < 0.05$ versus results for CLR-susceptible, MNZ-susceptible group; +++, $P < 0.001$ versus results for CLR-susceptible, MNZ-susceptible group; #, $P < 0.05$ versus results for CLR-resistant, MNZ-susceptible group.

We amplified the bacterial DNA by PCR and sequenced the *PBP1* genes in 30 strains between September 2008 and April 2010 (forward, 5′-CACRAGCACCGGTAAGATTT-3′; reverse, 5′-GCGACAATAAGAGTGGCA-3′). The sequences obtained were compared with the published sequences of *H. pylori PBP1* (L26695; GenBank accession number AE000511). Table 2 shows the substitutions detected in AMX-resistant strains. Strains with high-level resistance to AMX had 1 to 3 substitutions, and low-level-resistant strains (MICs of 0.06 to 0.25 μg/ml) had 0 to 2 substitutions. The accumulation of *PBP1* mutations could result in a gradual increase in AMX resistance. The $Asn_{535} \rightarrow Asp$ substitution was also detected in not only AMX-resistant strains but also 3 of 15 (20%) AMX-susceptible strains.

The AMX resistance rates were 13.6% (6/45) in the strains susceptible to both CLR and MNZ, 32.2% (66/205) in the strains resistant to CLR but susceptible to MNZ, 45.5% (5/11) in the strains resistant to MNZ but susceptible to CLR, and 48.8% (40/82) in the strains resistant to both CLR and MNZ. The AMX resistance rate in the strains resistant to CLR or MNZ was significantly higher than that in the strains susceptible to both CLR and MNZ. The rate of resistance to AMX in the strains resistant to both CLR and MNZ was significantly higher than that in the strains susceptible to MNZ (Table 3). Efflux pump systems in bacteria, which can eject drugs and toxic compounds, including antibiotics, have a critical role in the development of multidrug resistance. We recently reported that the expression of the TolC efflux pump (*hefA*) was significantly increased under MNZ exposure (14). The efflux pump of *H. pylori* is also associated with the development of resistance to CLR, in addition to 23S rRNA point mutations (2). In addition to the known mutations in the gene coding for PBP, activated efflux systems may also play a role in *H. pylori* resistance to AMX.

In conclusion, contrary to our expectations, resistance to AMX in *H. pylori* was gradually induced after unsuccessful eradication attempts. The data are clearly consistent with the association of resistance rates and eradication failures. If AMX-resistant *H. pylori* strains were to spread further, serious problems would arise, resulting in increasing eradication failures (10). Our results suggest that clinicians should be aware of

AMX resistance together with resistance to other antibiotics in the future.

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ORIGINAL RESEARCH COMMUNICATION

Two Amino Acids Mutation of Ferric Uptake Regulator Determines *Helicobacter pylori* Resistance to Metronidazole

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Abstract

Metronidazole (Mtz) is a prodrug that is converted to its active form when its nitro group is reduced and superoxide radicals are generated. The superoxide radicals are directly toxic to the bacterium. On the other hand, the transcriptional regulator, ferric uptake regulator (Fur), of *Helicobacter pylori* is a direct suppressor of the iron-cofactored superoxide dismutase SodB, which is essential for protection against superoxide attack. Here, we demonstrate that in some Mtz-resistant strains, SodB activity is induced in a dose-dependent manner on exposure to Mtz. Further, under Mtz exposure, the generation of superoxide radicals in Mtz-resistant strains was significantly reduced as compared with that in the Mtz-susceptible strains. These Mtz-resistant strains were found to carry amino acids mutation of Fur (C78Y, P114S; mutant-type Fur). The binding affinity of the mutant-type Fur to an operator sequence on the *sodB* promoter (Fur-Box) was significantly reduced. Our approach demonstrated that SodB expression is derepressed by mutant-type Fur, which is associated with the development of Mtz resistance. *Antioxid. Redox Signal.* 14, 15–23.

Introduction

HELICOBACTER PYLORI IS A GRAM-NEGATIVE BACTERIUM that colonizes the gastric mucosa in more than half of the entire population of the world; it is a major cause of chronic active gastritis and peptic ulcer disease and also an early risk factor for gastric cancer (16, 43). Eradication of this bacterium from the stomach results in recovery from gastritis and peptic ulcer disease in over 90% of patients. Metronidazole (Mtz) was initially used against a variety of anaerobic microorganisms, but the drug was later found to also exhibit activity against certain microaerophilic organisms such as *H. pylori*. Currently, one of the most effective treatment regimens for *H. pylori* consists of a combination of a proton pump inhibitor and any two of the following three antimicrobial agents: amoxycillin, Mtz, and clarithromycin (15).

Recently, a gradually increasing prevalence of Mtz resistance has begun to be reported from Asia and Europe (11, 26, 47). Kim *et al.* suggested that Mtz is also widely prescribed for other infections such as parasitic or genital infections and that such widespread use and abuse of this inexpensive drug may contribute to the increasing prevalence of Mtz resistance (26). This increase in the prevalence of Mtz resistance is likely to become an issue of concern in the clinical management of *H. pylori* infection. Mtz enters the cells by diffusion, and its antimicrobial toxicity is dependent on the reduction of its nitro group to nitro anion radicals and the generation of superoxide radicals (37, 38). According to Goodwin *et al.*, since nicotinamide adenine dinucleotide phosphate (reduced form) nitroreductase (RdxA) of *H. pylori* reduces the nitro group of Mtz to anion radicals that produce DNA strand breaks and oxidative stress, which ultimately cause rapid cell death (14), mutational inactivation of the *rdxA* gene would be expected to be associated with the development of resistance to Mtz. However, a number of Mtz-resistant strains have been reported in which the RdxA protein appears to be unchanged (23, 45, 49). In addition, Masaoka *et al.* has also isolated Mtz-resistant strains with an intact RdxA protein (31). These reports strongly suggest the existence of a resistance mechanism in the organisms other than RdxA inactivation. In the Mtz-resistant strains, superoxide radicals are generated through the reduction of Mtz; therefore, we focused on the radical scavenging activity in these Mtz-resistant strains.

H. pylori expresses only a single superoxide dismutase (SOD), the iron-cofactored superoxide dismutase (SodB) protein, which exhibits 53.5% identity to the *Escherichia coli* FeSod (41). SodB, as the primary defense against superoxide radicals, prevents interaction between iron and superoxide as

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well as catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. In addition, expression of SodB is also essential for gastric colonization by *H. pylori* and for its growth under microaerobic conditions (40).

Recently, Ernst *et al.* reported that *sodB* expression in *H. pylori* is directly regulated by the ferric uptake regulator (Fur) protein. Fur functions as a global transcriptional regulator and is involved in acid tolerance, detoxification of reactive oxygen species (ROS), and energy metabolism in *H. pylori* (5, 7, 12, 29). It is reported that Fur binds to iron (Fe^{2+}) and that the genes for iron uptake are repressed by the iron-binding form of Fur (10, 48). On the other hand, *sodB* expression is known to be repressed by the iron-free form of Fur (apo-Fur) (13). Apo-Fur binds to a specific consensus sequence called the Fur-Box located on the *sodB* promoter and blocks the binding of RNA polymerase (2, 13, 46).

In the present study, we attempted to confirm the hypothesis that Mtz-resistant strains which show no evident change of the RdxA protein exhibit an enhanced ability to defend themselves against superoxide radicals by SodB. The present study was designed to examine the expression of SodB and the structure and functions of Fur, which acts as a *sodB* transcriptional repressor, in Mtz-resistant strains.

Materials and Methods

Bacterial strains and culture conditions

H. pylori strains ATCC700392, KS0163, and KS0189 were used as the Mtz-susceptible strains; and strains KS0033, KS0048, and KS0145 were used as the Mtz-resistant strains. None of these Mtz-resistant strains showed any evident changes of the RdxA protein as determined by amino acid alignment analysis of the RdxA protein (31). According to the report of Masaoka *et al.*, KS0033 and KS0048 showed a moderate-level resistance (16 $\leq$ minimum inhibitory concentration [MIC] $<$32 μg/mL), and KS0145 showed a high-level resistance (32 μg/mL $\leq$MIC) to Mtz (31). In this study, KS strains isolated from patients were maintained at -80°C in Brucella Broth (Becton-Dickinson) containing 25% (vol/vol) glycerol. The bacteria were cultured on Columbia HP agar (Becton-Dickinson) for 2 days at 37°C, under microaerobic conditions maintained with AnaeroPack MicroAero (Mitsubishigas).

Total RNA isolation and quantitative RT-polymerase chain reaction

Since Fur activity is dependent on the concentration of iron in the medium, the bacteria, normalized to an OD_{600} of 0.5, were incubated with 0, 0.01 and 0.05 μg/mL Mtz for 3 h in an iron-free medium (saline). The total RNA of the bacteria incubated with Mtz (Sigma) was isolated using the SV Total RNA Isolation system (Promega). The reverse transcription (RT) reaction was performed using the PrimeScript RT reagent Kit (Takara), in accordance with the manufacturer's guidelines. For real-time polymerase chain reaction (PCR), the PCR amplification was performed using the SYBR Premix Ex Taq Perfect Real Time kit (Takara) in a Thermal Cycler Dice Real Time System (Takara). The primer sequences used were as follows: *sodB* mRNA: forward 5′-CGACTGCCCTAAGC GATG and reverse 5′-CCAATTCCAACCAGAGCC; the 16S rRNA gene mRNA primers have been previously described in detail (35). The *H. pylori* 16S rRNA gene was used as the internal control for the quantitative RT-PCR.

Measurement of SOD activity

Since the Fur activity is dependent on the concentration of iron in the medium, the bacteria, normalized to an OD_{600} of 0.5, were incubated with 0, 0.05, and 0.5 μg/mL Mtz for 5 h in an iron-free medium (saline). After sonication (1.5 min at 25% power) of the bacteria incubated with Mtz, the resultant bacterial lysates were centrifuged, and the SOD activities were measured using an SOD Assay Kit-WST (Dojindo), in accordance with the manufacturer's guidelines.

Electron spin resonance assay

A spin trapping agent, 5 μM 4-Hydroxy-TEMPO (Sigma) or 40 mM CYPMPO (Radical Research), was added to the bacteria, normalized to an OD_{600} of 0.5, and incubated with 0, 0.05 or 0.5 μg/mL Mtz for 5 h. After sonication of the bacteria, the resultant bacterial lysates were transferred to a quartz flat cell (disposables) (Radical Research), and the radical intensity was determined by electron spin resonance (ESR) spectroscopy (JESRE1X, X-band; 100 kHz modulation frequency; Jeol) at 20°C.

Measurement of RdxA activity

After sonication (1.5 min at 25% power) of the bacteria cultivated for 2 days in the Brucella Broth plate, the resultant bacterial lysates were centrifuged, and the protein concentrations were measured using the BCA method (Pierce). RdxA activity was spectrophotometrically measured with reduction of Mtz observed at 320 nm. The reaction mixture contained Tris/acetate (100 mM Tris-HCl, 50 mM acetate), pH 7.0, 0.05 mM Mtz, and 0.3 mM nicotine adenine dinucleotide (reduced form), as described by Goodwin *et al.* (14).

Construction of SodB overexpression strain and rdxA deletion mutant strain

The shuttle vector pHel3 (19) was used as a scaffold to construct a SodB-overexpressing strain of *H. pylori*. The *sodB* gene was PCR-amplified with specific primers (forward 5′-CTCGAGATTAACTTTTAAAAAATTTAAAAAGAATTTG and reverse 5′-GGTACCTTAAGCTTTTTTATGCACC) and cloned into the pHel3 shuttle vector as a *Kpn*I-*Xho*I fragment. A nucleic acids sequencing of a *Kpn*I-*Xho*I fragment was performed on the pHelSodB construct, and then the construct was electroporated into *H. pylori*, which was grown on kanamycin to obtain a SodB-overexpressing strain. On the other hand, *H. pylori* transfected with only the pHel3 shuttle vector was grown on kanamycin to obtain the control strain.

The target-region gene cassette (5′*rdxA-chloramphenicol acetyltransferase* (*cat*)-3′*rdxA*) for construction of *rdxA* deletion mutant strain was cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA), and then the sequences were determined (target-vector). The target-vector was electroporated into *H. pylori* ATCC700392, which was grown on 20 μg chloramphenicol (Sigma) to obtain an *rdxA* deletion mutant strain of *H. pylori* ATCC700392.

Measurement of the MIC to Mtz

The bacteria (at an OD_{600} of 0.1) were inoculated on an agar plate containing Mtz in serial twofold dilutions (0.5–128 μg/mL). All the plates were incubated at 37°C under microaerobic conditions, and the MIC values were determined (32).

DNA sequencing and protein modeling of H. pylori *Fur*

The complete *fur* gene and the promoter region of *sodB* were PCR-amplified with specific primers (*fur* gene: forward 5′-ATGAAAAGATTAGAAACTTTG and reverse 5′-ACATT CACTCTCTTGGCATTCT; *sodB* promoter gene: forward 5′-CCCTTAAAATCCACAAAATTTGC and reverse 5′-GTA ATGTAAACATGTTTTCTCCTTGTG) using Ex Taq DNA polymerase (Takara). The PCR products were cloned into the pCR4-TOPO vector (Invitrogen), and then the sequences of the *fur* and *sodB* promoter genes were determined using the BigDye terminator V1.1 Cycle Sequencing Kit (Applied Biosystems); the deduced amino acids were then aligned using GENETYX Version 5.1. The protein structures were modeled and displayed using Swiss-Model (www.expasy.org/swissmod) and DeepView-Swiss-PdbViewer (www.expasy.org/spdbv/), respectively.

Expression and purification of H. pylori *Fur*

The *fur* gene was PCR-amplified with specific primers (ExFur gene: forward 5′-CATATGAAAAGATTAGAAACTT TGG and reverse 5′- AGATCTGGACATTCACTCTCTTG) and cloned into the pET-30b (+) (Novagen) as an *NdeI-BglII* fragment. The pETFur construct was transformed into *E. coli* BL21 (DE3), and the expression was achieved by induction, by the addition of 0.5 mM IPTG, of a 200 mL culture incubated for 6–8 h at 30°C and grown to an OD_{600} of 0.6. The Fur protein expressed in this strain as a C-terminal Six-His tagged protein was purified using the MagneHis Protein Purification System (Promega).

Apo-Fur binding analysis by surface plasmon resonance assay

A Biacore 2000 instrument (Biacore AB) was used to perform the Surface Plasmon Resonance assay in accordance with the manufacturer's guidelines. To construct the biotinylated *sodB* promoter gene of each strain, each *sodB* promoter gene was PCR-amplified with specific biotinylated primers (forward 5′-CCCTTAAAATCCACAAAATTTGC and reverse 5′-Bio-GTAATGTAAACATGTTTTCTCCTTGTG). To conduct the analyses under a low-iron condition, the following buffer was used for the analyses: HBS-EP running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid, 0.005% surfactant P20) and biotinylated PCR products of the *sodB* promoter were immobilized on to Sensor Chip SA (GE Healthcare). At least five concentrations of each purified Fur protein were applied to the *sodB* promoter-immobilized Sensor Chip SA in HBS-EP buffer at a flow rate of 10 μl/min. The response value of the reference cell (flow cell 3, blank) was subtracted from the response value of each flow cell 4 (*sodB* promoter) to correct for nonspecific binding. The data were analyzed, and the dissociation constant (*Kd*) values were calculated using a BIAevaluation software (Baicore).

Results

Expression of SodB under Mtz exposure

In the Mtz-susceptible strain ATCC700392, *sodB* mRNA expression was scarcely derepressed under Mtz exposure (Fig. 1a). On the other hand, in the Mtz-resistant strains, which showed no evident change of the RdxA protein (KS0033, KS0048, and KS0145), the *sodB* mRNA expression was derepressed in a dose-dependent manner under exposure to Mtz (Fig. 1a). Further, no increase of the SodB activity was observed in the Mtz-susceptible strain, whereas significant increase of the SodB activity was found in the Mtz-resistant strains in the presence of 0.5 μg/mL Mtz (Fig. 1b).

Generation of superoxide radicals in H. pylori *under Mtz exposure*

To assess whether ROS generation was suppressed by the overexpression of SodB in the Mtz-resistant strains, we measured the amount of ROS produced in each type of *H. pylori*

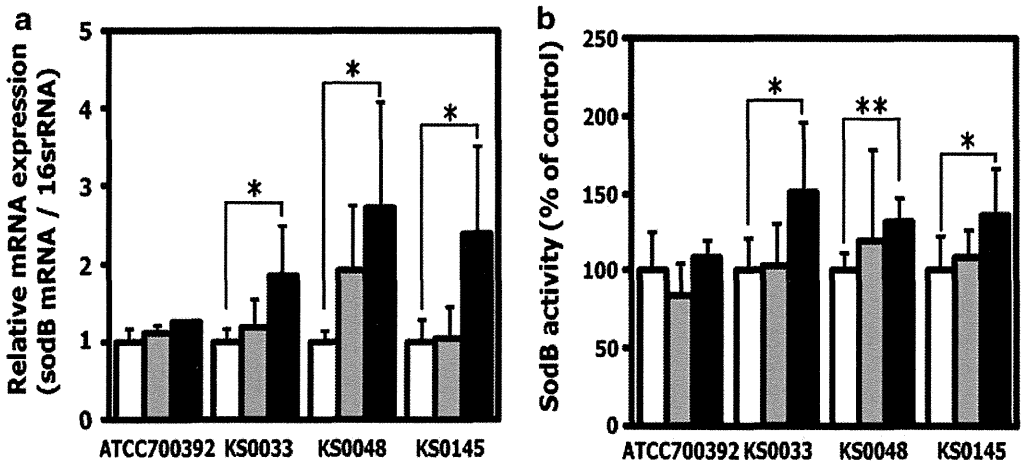

FIG. 1. Expression of SodB under Mtz exposure. (**a**) Expression of *sodB* mRNA in an Mtz-susceptible strain (ATCC700392) and Mtz-resistant strains (KS0033, KS0048, and KS0145) exposed to 0 (white), 0.01 (gray), and 0.05 (black) μg/mL Mtz was measured by quantitative reverse transcription–polymerase chain reaction. (**b**) Expression of SodB activity in an Mtz-susceptible strain (ATCC700392) and Mtz-resistant strains (KS0033, KS0048, and KS0145) exposed to 0 (white), 0.05 (gray), and 0.5 (black) μg/mL Mtz was measured by the method described in the Materials and Methods section. Results are means ± SD of three independent assays. Asterisks indicate statistical significance from each strain with no Mtz exposure, $^{*}p < 0.05$, $^{**}p < 0.01$. Mtz, metronidazole; SodB, iron-cofactored superoxide dismutase.

strain under Mtz exposure by ESR assay. Although significant dose-dependent increase in the generation of ROS was observed after exposure to Mtz in the Mtz-susceptible strains, the ROS generation was significantly reduced in the Mtz-resistant strains (Fig. 2a). Further, Figure 2b shows the presence of the superoxide radical-specific signal of ESR detected with the CYPMPO reagent in the Mtz-susceptible strain, whereas no such specific signals can be seen in the Mtz-resistant strains.

Effect of SodB overexpression on H. pylori *susceptibility to Mtz*

To assess the contribution of the SodB overexpression to Mtz resistance, a SodB-overexpressing strain was constructed using a pHel3 shuttle vector (19). The SodB activity of the SodB-overexpressing strain (ATCC700392 pHel3::*sodB*) was twofold higher as compared with that of the control strain (ATCC700392 pHel3 control) (data not shown). Although the MIC of Mtz for the ATCC700392 strain and pHel3 control strain was the same as that for the Mtz-susceptible strains (MIC $<8\,\mu g/mL$), the MIC values for KS0033, KS0048, KS0145, and ATCC700392 pHel3::*sodB* were 64, 32, 128, and $32\,\mu g/mL$, respectively (Table 1). Thus, these strains showed a high level resistance to Mtz (MIC $\geq 32\,\mu g/mL$). In addition, to assess the Mtz reduction activity associated with Mtz resistance of KS0033, KS0048, and KS0145, the RdxA activity was spectrophotometrically measured with reduction of Mtz at 320 nm. The RdxA activity for KS0033, KS0048, KS0145, ATCC700392 pHel3::*sodB*, and ATCC700392 pHel3 control were not decreased compared with ATCC700392 (Table 1). On the other hand, the RdxA activity of ATCC700392 Δ*rdxA*, which showed a moderate-level resistance to Mtz ($8 \leq$MIC $<32\,\mu g/mL$), was significantly decreased compared with ATCC700392 (Table 1). This result indicated that RdxA inactivation did not contribute to development of the Mtz resistance in the KS0033, KS0048, KS0145, and ATCC700392 pHel3::*sodB*. Therefore, these findings strongly suggest that SodB overexpression contributes to Mtz resistance in the KS0033, KS0048, KS0145, and ATCC700392 pHel3::*sodB*.

Alignment of the nucleic acid sequence of the SodB promoter and the amino acid sequence of Fur

To assess the mechanism of SodB overexpression in the Mtz-resistant strains, we focused on the regulation of *sodB* expression by Fur. We aligned the nucleic acid sequence of the *sodB* promoter (Fur-Box) and the predicted amino acid sequence of Fur for the Mtz-susceptible strains (ATCC700392, KS0163, and KS0189) and Mtz-resistant strains (KS0033, KS0048, and KS0145). The A-5C mutation of the Fur-Box was detected in all of the clinical isolates from Keio University hospital (Fig. 3a). Although KS0145 showed a G-3A mutation adjacent to the Fur-Box, no distinct mutation of the Fur-Box was observed in the Mtz-resistant strains (Fig. 3a). On the other hand, two distinct mutations of the amino acid sequence of Fur were noted in the Mtz-resistant strains (Fig. 3b). KS0145 had a mutant-type Fur protein, with Cys 78 replaced by Tyr (C78Y) and Asn 118 replaced by His (N118H). KS0033 and KS0048 also showed a mutant-type of Fur, with Pro 114 replaced by Ser (P114S) and N118H (Fig. 3b). The HHDHXXCXXC motif, which is believed to be involved in the binding of the iron cofactor, was highly conserved (Fig. 3b) (4).

Kd value of apo-wild-type Fur and apo-mutant-type Fur

To assess the effect of the amino acid mutations of Fur (mutant-type Fur) on the affinity of apo-Fur for the Fur-Box, we examined the affinity of each of the apo-Fur proteins for the *sodB* promoter (Fur-Box) by Surface Plasmon Resonance assay (Biacore 2000). Beforehand, it was confirmed that the *Kd* value of apo–wild type (WT)-Fur to Fur-Box was similar to the value that Ernst *et al.* reported (13), and then the *Kd* value of

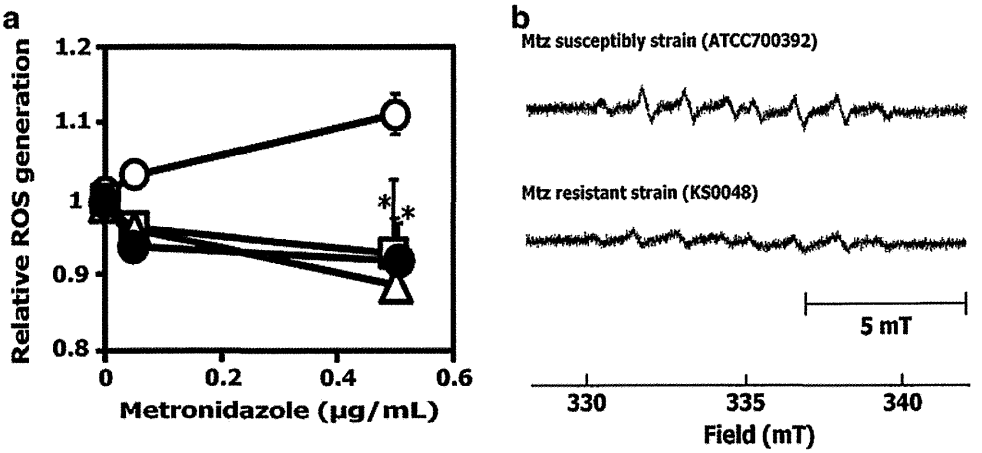

FIG. 2. Generation of superoxide radicals under Mtz exposure. (**a**) The induction of ROS was measured by electron spin resonance using 5 μM 4-Hydroxy-TEMPO in an Mtz-susceptible strain (ATCC700392) (white circle) and Mtz-resistant strains (KS0033 [white square], KS0048 [black circle], and KS0145 [white triangle]) exposed to 0, 0.05, and 0.5 μg/mL Mtz. The ROS generation was calculated as reference in the ROS generation of each strain without Mtz exposure. Results are means ± SD of three independent assays. Asterisks of KS0048 and KS0145 indicate statistical significance for the comparison with Mtz-susceptible strain (ATCC700392) as determined by Student's *t*-test (*$p < 0.05$). (**b**) Representative signal patterns of generation of superoxide radicals in the Mtz-susceptible stain and Mtz-resistant strains exposed to 0.5 μg/mL Mtz as measured by electron spin resonance using 40 mM CYPMPO. ROS, reactive oxygen species.

TABLE 1. THE EFFECT OF SUPEROXIDE DISMUTASE-OVEREXPRESSION AND RdxA ACTIVITY ON MINIMUM INHIBITORY CONCENTRATION (μG/mL) OF METRONIDAZOLE

Strains	*RdxA activity (nmol/min/mg protein)*	p-*Value*	*Minimum inhibitory concentration (μg/mL)*	*Metronidazole susceptibility*
ATCC700392	2.57 ± 0.26		2	Susceptible level
ATCC700392 Δ*rdxA*	1.29 ± 0.19	<0.01	16	Moderate level resistance
KS0033	2.45 ± 0.41	0.59	64	High level resistance
KS0048	2.35 ± 0.08	0.11	32	High level resistance
KS0145	2.40 ± 0.23	0.23	128	High level resistance
ATCC700392 pHel3::sodB	2.39 ± 0.05	0.16	32	High level resistance
ATCC700392 pHel3 control	2.77 ± 0.05	0.23	4	Susceptible level

SodB, iron-cofactored superoxide dismutase; RdxA, nicotinamide adenine dinucleotide phosphate (reduced form) nitroreductase.

apo-mutant-type Fur to Fur-Box as control with that of apo-WT-Fur was measured. The results of the assay revealed a significant increase of the *Kd* value for the apo-mutant-type Fur in the Mtz-resistant strains as compared with that of apo-WT-Fur in the Mtz-susceptible strains (Fig. 4). These results indicate a significantly decreased affinity of apo-mutant-type Fur for the Fur-Box and that the SodB expression in the Mtz-resistant strains is not repressed to the same extent as that in the Mtz-susceptible strains (Fig. 5).

Further, to assess the effect of nucleic acid mutations of the *sodB* promoter on the affinity of apo-Fur for the Fur-Box, we examined the affinity of apo-ATCC700392 Fur for the KS0145

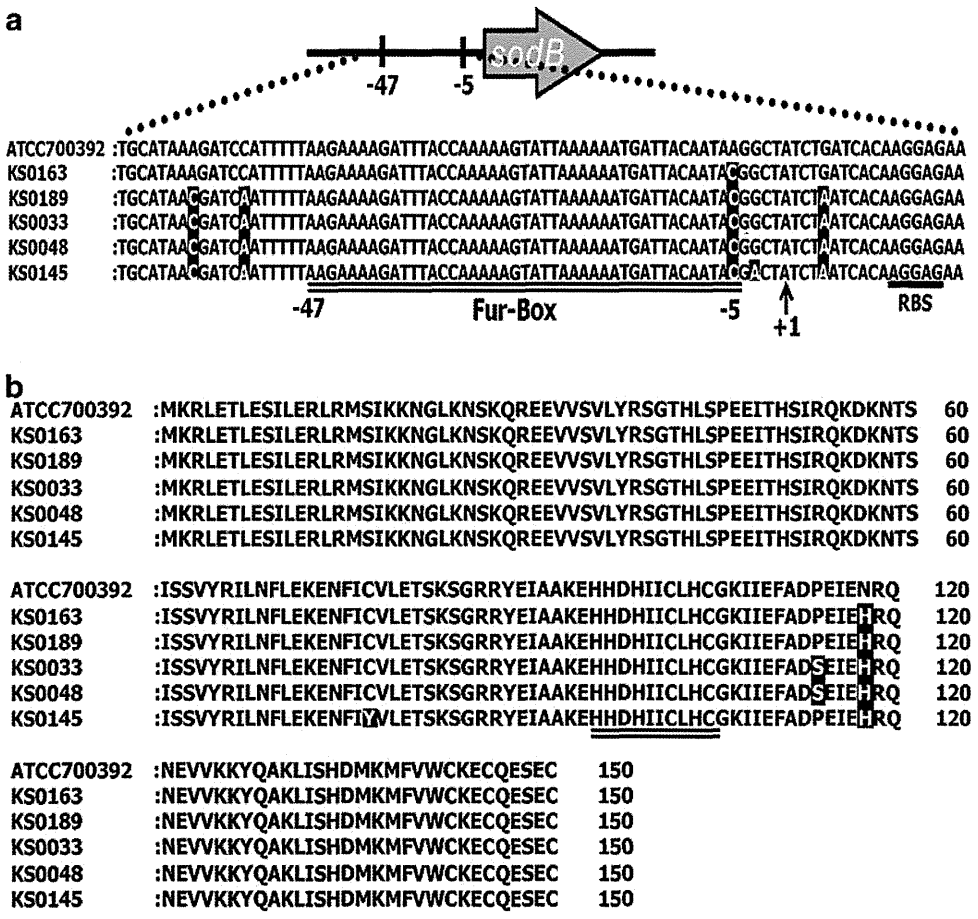

FIG. 3. Alignments of the *Helicobacter pylori sodB* promoter and Fur protein. (**a**) Alignment of the *sodB* promoter from the Mtz-susceptible strains (ATCC700392, KS0163, and KS0189) and Mtz-resistant strains (KS0033, KS0048, and KS0145). Each mutation point is marked in white. The predicted Fur-Box ranges from −5 to −47 and is indicated by the double line. +1 indicates the *sodB* transcriptional start site, and RBS indicates the ribosmal binding site. (**b**) Alignment of the predicted Fur amino acid sequences of Mtz-susceptible strains (ATCC700392, KS0163, and KS0189) and Mtz-resistant strains (KS0033, KS0048, and KS0145). Each mutation point is marked in white. The highly conserved motif (HHDHXCXXC) believed to be involved in the binding of the iron cofactor is indicated by the double lines. Fur, ferric uptake regulator.

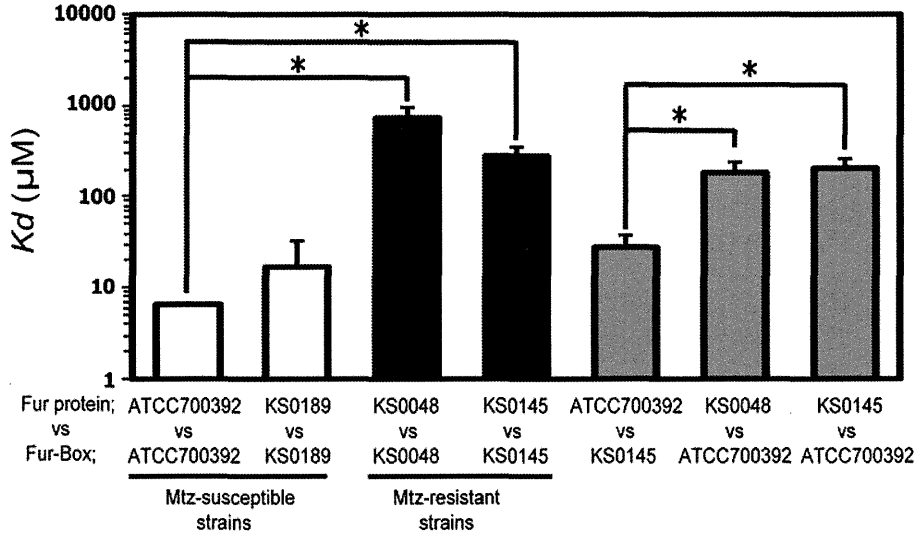

FIG. 4. Mutation of Fur affects its affinity for the Fur-Box. The *Kd* value for binding of each apo-Fur protein to each Fur-Box was calculated as reference in the Flow Cell in which *sodB* promoter was not immobilized on to Sensor Chip SA using a BIAevaluation software, and the combination of apo-Fur protein and Fur-Box is denoted as Fur protein *versus* Fur-Box. White bar indicates the affinity of apo–wild type (WT)-Fur for the Fur-Box of the Mtz-susceptible strains, black bar indicates the affinity of apo-mutant-type Fur for the Fur-Box in the Mtz-resistant strains, and the gray bar indicates the effect of the nucleic acid mutations of the Fur-Box on the affinity of apo-Fur for the Fur-Box. Results are means ± SD of three independent assays. Asterisks indicate statistical significance from using an apo-WT-Fur, $*p < 0.05$. *Kd*, dissociation constant.

Fur-Box and the affinity of apo-mutant-type Fur for the ATCC700392 Fur-Box. The *Kd* value of apo-ATCC700392 Fur for binding to the KS0145 Fur-Box was fourfold higher as compared with that for the binding to the ATCC700392 Fur-Box, although the difference was not significant (Fig. 4). On the other hand, the *Kd* values of apo-mutant-type Fur for binding to the ATCC700392 Fur-Box were scarcely reduced as compared with that for its binding to the KS0145 or KS0048 Fur-Box (Fig. 4). The results of the assay revealed a significant increase of the *Kd* values of apo-mutant-type Fur for binding to the ATCC700392 Fur-Box as compared with that of apo-ATCC700392 Fur for binding to the KS0145 Fur-Box (Fig. 4).

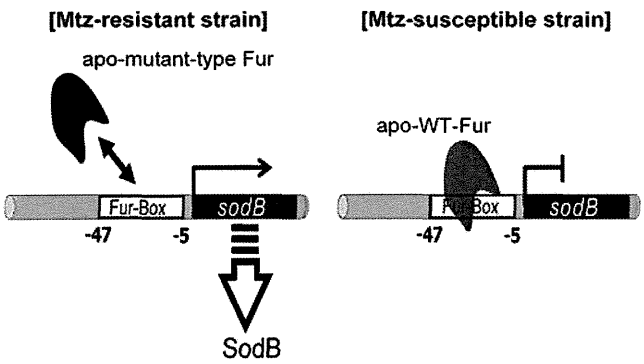

FIG. 5. Schematic representation of the proposed mode of action of apo-mutant type-Fur in the Mtz-resistant strains and apo-WT-Fur in the Mtz-susceptible strains. The apo-Fur binds to an operator sequence called Fur-Box in the *sodB* promoter, and then binding of apo-Fur suppresses *sodB* expression. The affinity of the apo-mutant-type Fur to the Fur-Box is significantly decreased, and then *sodB* expression of Mtz-resistant strains is more derepressed than Mtz-susceptible strains.

Prediction of the three-dimensional structure of H. pylori Fur

To predict the positions of the mutations in the three-dimensional structure of Fur, the structure was determined using a Swiss Model and DeepView-Swiss-PdbViewer. The N-terminal domain possessing four helices followed by a loop was formed by the residues located between two antiparallel β-strands. The C-terminal domain, which was separated by a coil from the N-terminus possessing two antiparallel β-strands, was followed by another β-strand located between the two helices (Fig. 6). C78Y is predicted to belong to a β strand in the N-terminal domain, whereas P114S and N118H are predicted to belong to a C-terminal domain (Fig. 6).

Discussion

The present study revealed amino acid mutations of Fur in some Mtz-resistant strains with the RdxA activity remaining with reduced affinity of the mutant Fur for the Fur-Box, and enhancement of the superoxide radical scavenging activity in these strains, as *sodB* was not repressed to the same extent by the apo-mutant-type Fur in these strains as by the wild-type apo-fur in the Mtz-susceptible strains (Figs. 1–4, Table 1).

Recently, Carpenter *et al.* reported that the A-5C mutation of the Fur-Box decreases the affinity of apo-Fur for the Fur-Box in *H. pylori* (6). In the present study, the A-5C mutation of the Fur-Box was detected in all of the tested clinical isolates (Fig. 3a). In the Surface Plasmon Resonance assay, the *Kd* value for the binding of apo-ATCC700392 Fur to the KS0145 Fur-Box was fourfold higher as compared with that for its binding to the ATCC700392 Fur-Box (Fig. 4), suggesting that the A-5C mutation in the Fur-Box is important for the binding with apo-ATCC700392 Fur, which is consistent with the report of Carpenter *et al.* (6). On the other hand, in the Mtz-resistant strains, the A-5C mutation hardly influenced the interaction between

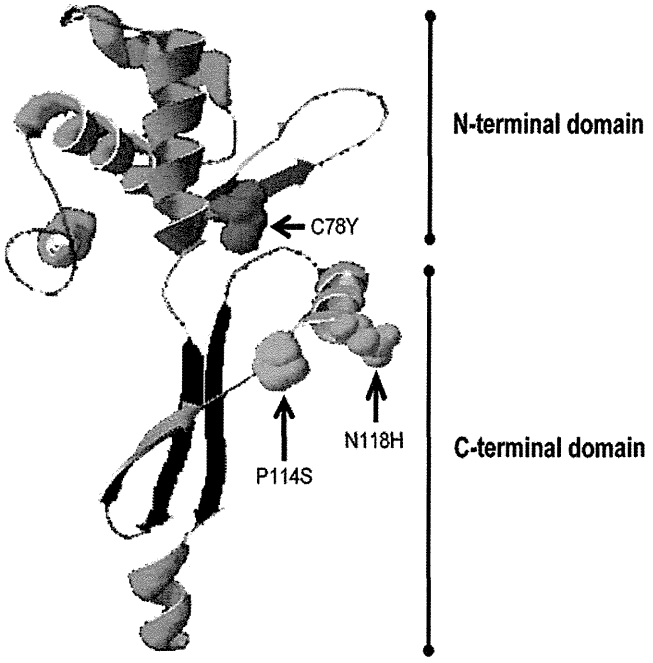

FIG. 6. Prediction of the three-dimensional structure of the Fur protein. Each mutation point is marked with an arrow. C78Y is predicted to exist in the N-terminal domain, whereas P114S and N118H are predicted to be located in the C-terminal domain. The three-dimensional structure was determined using a Swiss-Model and DeepView-Swiss-PdbViewer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the Fur-Box and the apo-mutant-type Fur (Fig. 4). These results indicate that the Fur mutations C78Y, P114S, and N118H could play a greater role on the affinity of apo-Fur for the Fur-Box than the A-5C mutation in the Fur-Box.

The Fur protein has been best characterized in *E. coli*, in which it has been shown to possess three functional domains (the DNA-binding domain, iron-binding domain, and the oligomerization domain), and the protein binds to the Fur-Box after dimerization (17, 36, 42, 46). The Fur monomer of *E. coli* has been reported to consist of a helix-turn-helix motif and two β strands separated by a turn that forms the wings on the N-terminal domain, which is considered to be involved in the DNA binding (21, 42, 46). On the other hand, the C-terminal domain of *E. coli* Fur, separated by a coil from the N-terminal, consists of two antiparallel β-strands, which are considered to be involved in the oligomerization of the protein (21, 42). From the results of the homology modeling of *H. pylori* Fur, it was inferred that *H. pylori* Fur also has three functional domains (the DNA-binding domain near the N-terminal, iron cofactor-binding domain (HHDHXXCXXC), and the oligomerization domain near the C-terminal) (4). Therefore, it was inferred that the C78Y mutation of the KS0145 strain was located in the DNA-binding domain and that the P114S and N118H mutations of KS0033 and KS0048 strains were located in the oligomerization domain using a homology modeling (Fig. 6). Therefore, these mutations are predicted to affect the affinity of the Fur protein for the Fur-Box. However, the amino acid sequence of *H. pylori* Fur exhibited moderate identity (23%–37%) to the Fur protein from other bacteria present in the database, such as *Campylobacter jejuni*, *E. coli*, *Haemophilus influenzae*, *Vibrio cholerae*, *Bordetella pertussis*, *Klebsiella pneumoniae*, *Neisseria meningitides*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, suggestive of a moderate homology (4). This finding indicates that the amino acids which are important for DNA binding or dimerization may differ between *H. pylori* Fur and other bacterial Fur proteins.

ROS damage of pathogenic bacteria constitutes a key part of the immune response of the host. Many studies have shown that *H. pylori* infection elicits a strong oxidative stress response from the host (1, 3, 9, 44). To survive the effects of production of ROS by the host, *H. pylori* depends on a significant repertoire of detoxification enzymes, such as SodB, catalase (KatA), and neutrophil-activating protein (NapA) (18, 34, 41). Upstream of *katA*, a low-affinity putative Fur-Box has been identified (30, 33). In addition, Cooksley *et al.* reported that Fur is involved in *napA* regulation and that a potential Fur-Box by which this control could be mediated has been identified (8). Accordingly, the expression of *katA* and/or *napA* might be derepressed by mutant-type Fur, leading to enhancement of the ability of *H. pylori* to colonize the human stomach.

In the present study, we demonstrated that the overexpression of SodB mediated by mutant-type Fur may underlie the RdxA-independent resistance of *H. pylori* to Mtz. Recently, it has come to be recognized that in addition to RdxA, some other proteins such as pyruvate oxidoreductase, nicotinamide adenine dinucleotide phosphate (reduced form) flavin oxidoreductase (FrxA), and ferredoxin-like protein (FdxB) may also be associated with the activation of Mtz (22, 25). Many researchers have demonstrated an association between inactivation of these proteins and resistance to Mtz (20, 24, 27, 28). On the other hand, Jenks *et al.* reported that RdxA-independent mechanisms may play only a relatively minor role in Mtz resistance or may be involved only in the transition to high-level resistance (22). Although it is difficult to determine whether overexpression of SodB associated with mutant-type Fur entirely accounts for RdxA-independent Mtz resistance, it is, nevertheless, an important mechanism that participates in not only Mtz resistance but also resistance of the host immune responses to ROS.

Recently, overexpression of Fe-SOD was reported to be associated with the Mtz resistance in Mtz-resistant strains of the protozoan parasite *Entamoeba histolytica*, which is the causative agent of human amoebiasis (39, 50). Based on these reports, it is considered that overexpression of SOD may affect the Mtz resistance mechanism in many bacterial species.

In conclusion, the present study demonstrates a novel mechanism of Mtz resistance of *H. pylori*, namely, aberrant increase of SodB expression resulting from mutations of Fur.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ESR = electron spin resonance
Fur = ferric uptake regulator
Kd = dissociation constant
Mtz = metronidazole
PCR = polymerase chain reaction
RdxA = nicotinamide adenine dinucleotide phosphate (reduced form) nitroreductase
ROS = reactive oxygen species
SodB = iron-cofactored superoxide dismutase